Transcriptome Analysis of the Zebrafish Pineal Gland

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The zebrafish pineal gland (epiphysis) is a site of melatonin production, contains photoreceptor cells, and functions as a circadian clock pace maker. Here, we have used microarray technology to study the zebrafish pineal transcriptome. Analysis of gene expression at three larval and two adult stages revealed a highly dynamic transcriptional profile, revealing many genes that are highly expressed in the zebrafish pineal gland. Statistical analysis of the data based on Gene Ontology annotation indicates that many transcription factors are highly expressed during larval stages, whereas genes dedicated to phototransduction are preferentially expressed in the adult. Furthermore, several genes were identified that exhibit day/night differences in expression. Among the multiple candidate genes suggested by these data, we note the identification of a tissue-specific form of the *unc119* gene with a possible role in pineal development. *Developmental Dynamics 238:1813–1826, 2009.* © 2009 Wiley-Liss, Inc.

Key words: pineal gland; epiphysis; zebrafish; circadian rhythm; microarray

Accepted 11 April 2009

INTRODUCTION

The pineal gland (epiphysis) is located at the dorsal edge of the diencephalon in the zebrafish. The conserved function of this organ in all vertebrates is the synthesis and secretion of melatonin, a hormone that regulates a variety of circadian and circannual physiological processes (Arendt, 1995; Falcon, 1999; Klein, 2004). Melatonin levels are high at night and low during the day, as a consequence of regulated transcription and stability of serotonin-N-acetyltransferase (AANAT), the rate-determining enzyme of melatonin synthesis. In zebrafish and certain other non-mammalian vertebrates, the melatonin producing cells of the pineal gland are photoreceptors that can rhythmically produce melatonin for several days in isolation, reflecting the presence of an autonomous circadian clock pacemaker within these photoreceptor cells (Bernard et al., 1997; Begay et al., 1998; Falcon, 1999). Therefore, the fish pineal photoreceptor cell is a valuable model system to study circadian function, photodetection, and melatonin production.

In addition, the zebrafish pineal gland is the first site where neurogenesis occurs, being apparent at approximately 24 hours post fertilization (hpf; Chitnis and Kuwada, 1990; Wilson and Easter, 1991). The existence of neuronal cells in the pineal gland which send projections to the brain makes this tissue more heterogeneous as compared to the pineal gland of mammals (Masai et al., 1997). The neuronal patterning surrounding the pineal gland is regulated by the homeobox transcription factor *floating head* (*flh*) and by *masterblind* (*mbl*), which encodes the negative regulator of *wnt* signaling Axin. $Flh^{-/-}$ ze-

Additional Supporting information may be found in the online version of this article.

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DOI 10.1002/dvdy.21988
Published online 29 May 2009 in Wiley InterScience (www.interscience.wiley.com).

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brafish show reduced neuronal production in the pineal gland, whereas mutations in *mbl* increase the number of pineal neurons throughout the dorsal forebrain (Masai et al., 1997). Furthermore, the basic helix loop helix (bHLH) transcription factors *achaete/scute homologue 1a* (*ascl1a*) and *neurogenin1* (*ngn1*) act downstream of *flh* to regulate neurogenesis (Cau and Wilson, 2003). The molecular mechanisms of pineal gland development and function beyond these initial steps of neurogenesis have not been fully explored. Recently, pineal development and its relationship to brain asymmetry has received considerable attention (Gamse et al., 2003, 2005; Halpern et al., 2003; Aizawa et al., 2005, 2007; Hendricks and Jesuthasan, 2007; Kuan et al., 2007a,b). Asymmetry depends on the laterality of the parapineal and is controlled by Nodal signaling (Concha et al., 2000, 2003; Liang et al., 2000).

Gene profiling of the pineal gland of the chicken and rat have identified many genes that are highly expressed in the pineal gland, show night/day differences, or both (Humphries et al., 2002; Bailey et al., 2003, 2008; Fukuhara and Tosini, 2008). Here we report the results of global transcriptome analysis of the zebrafish pineal gland taken from day 3 larvae to adults. Many highly abundant transcripts that had not been previously reported to be present in this tissue have been identified. Furthermore, many highly expressed genes were found to dynamically change their expression levels during development. These data provide a broad basis for further molecular analysis of pineal gland development and physiology.

RESULTS

As a first step in data analysis, all pineal gland data were averaged including data obtained during the day and night and at all five developmental stages; brain data were treated similarly. Probe sets were selected with the following criteria: P value ≤ 0.05 and pineal/brain signal ratio ≥ 5. Among the total 15,503 probe sets in the globally averaged data pool, 94 met these criteria. Of these, 43 probe sets have been annotated (http://www.affymetrix.com/analysis/index.affx), and nearly half of them (21 probe sets) correspond to genes known to be highly expressed in the pineal gland, including *aanat2* (Gothilf et al., 1999), *floating head* (Talbot et al., 1995), *extra-ocular rhodopsin* (Mano et al., 1999), *phosducin* (accession number XM_677731), *Crx* (Liu et al., 2001), and *otx5* (Gamse et al., 2002). Expression of GFP in the pineal gland of the transgenic fish used in this study was confirmed. These observations provide a first-level indication that our data effectively discriminate between genes that are differentially expressed in the pineal gland and the brain. Furthermore, principle component analysis of individual repeats indicated that the data are of high quality (not shown).

Genes Highly Expressed in the Pineal Gland Relative to Brain

Genes were considered to be highly expressed in the pineal gland relative to brain if the probability of a difference was ≤ 0.05. Setting the absolute difference at greater than threefold changes the number of selected genes (more accurately probe sets) shown in Table 1. The number of genes highly expressed in the pineal gland identified in this manner is much higher in adults than in larvae, possibly reflecting functional maturation of the tissue. Similar numbers of transcripts were enriched in the samples collected during the day and night. Probe sets selected at a threefold criterion in the three larval stages or the two adult stages partially overlapped, as shown in Figure 1. Approximately 20–35% of the transcripts detected at each stage were not detected at other stages, while approximately 60% of probe sets overlapped in RNA samples of 3 month and 1- to 2-year-old zebrafish. The total number of nonoverlapping probe sets at larval stages was 128 during the day and 150 at night, while 1,018 and 1,017 nonoverlapping probe sets were selected in adult day and night samples, respectively. Approximately 60% of probe sets identified as highly expressed in the larval pineal gland were also found to be enriched in adult tissue.

We next compared the results of day and night analysis. Among the 128 genes highly expressed in the larval pineal gland relative to the brain at day and the 150 genes highly expressed at night, 83 were expressed both at day and night. Because more than 1,000 probe sets were selected as pineal enriched transcripts in adult samples under these criteria, we applied more stringent criteria to select genes. Increasing the required pineal/brain difference from three- to fivefold while maintaining all other conditions, the numbers of nonoverlapping probe sets highly expressed in adult (3 month and 1–2 years old) pineal glands were 322 and 365 at day and night, respectively; among these, 197 were expressed during both day and night.

The 50 annotated genes most highly enriched in the pineal gland in at least one of the stages studied are presented in Table 2 (larvae) and Table 3 (adult). More complete information on the genes selected by our criteria at larval and adult stages are listed in Supplementary Table S1 (3× enrichment, which is available online) and Supplementary Table S2 (5× enrichment). These lists include many more genes highly expressed in the pineal gland than previously identified. Many of these genes code for proteins involved in photoreceptor signal transduction pathways including G proteins and cGMP specific phospho-

TABLE 1. Number of Genes Enriched in the Pineal Gland as Compared to Brain (P/B), Listed at Different Enrichment Ratios[a]

	d3	d5	d10	3mo	1-2 yr
Day					
P/B					
≥3	100	64	56	751	724
≥5	50	37	28	385	363
≥10	21	19	17	155	194
Night					
P/B					
≥3	102	70	91	878	566
≥5	53	35	49	432	245
≥10	23	19	21	191	123

[a]Samples were analyzed at midday and midnight at each developmental stage. The following criteria were used for the selection of probe sets. Average minimum signal ≥ 200; P value ≤0.05.

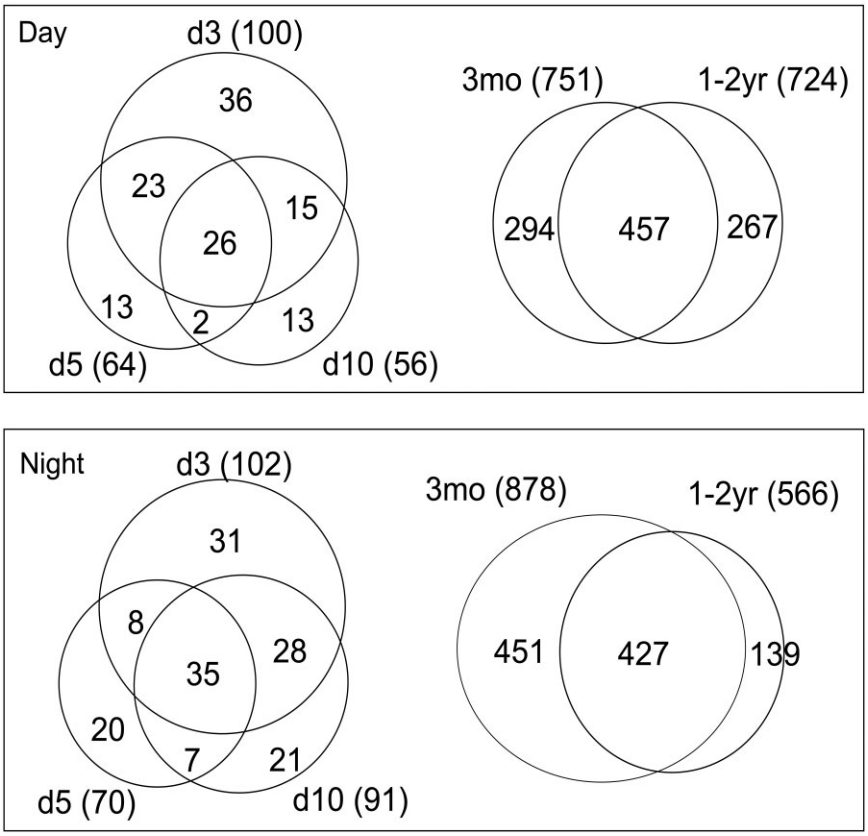

Fig. 1. Overlap of pineal-enriched genes at different stages. Venn diagrams of genes enriched in the pineal gland threefold or higher compared with the brain at different stages; larval and adult stages are shown separately. The total number of nonoverlapping enriched genes is 128 (larvae, day), 150 (larvae, night), 1,018 (adult, day), 1,017 (adult, night).

diesterase. We also identified a homolog of *unc119*, *rbp4* (retinol binding protein 4), *zic* family members, and *iroquois* homeobox proteins as transcripts that are pineal enriched as compared to the brain. The *unc119* homolog was studied further (see below).

At least two habenula-specific genes, *cadps2* and *leftover*, were also identified as pineal enriched genes (Table 2, #32 and #36, respectively). Because habenular nuclei are located very close to the pineal gland at larval stages, this may be due to contamination of the pineal sample with habenular tissue. As an independent approach to test the quality of the analysis, we picked a random series of unannotated clones and analyzed their expression pattern by in situ hybridization. Twelve of 19 clones analyzed showed enhanced expression in the pineal gland relative to the surrounding brain (Supp. Fig. S1, which is available online), while the remaining clones, whose expression levels were moderate to weak in the microarray analysis, were not detected by in situ hybridization (Supp. Fig. S1).

Analysis of Genes Highly Expressed in the Pineal Gland

To obtain functional insights into the categories of genes highly expressed in the pineal gland, Gene Ontology (GO) analysis was done using human homologs, as described in the Experimental Procedures section. Human homologs could be identified for 43–50% of zebrafish probe sets annotated for the Affymetrix Genechip. Approximately 50% of zebrafish probe sets are not well annotated (referred to as "transcribed loci" or "hypothetical proteins") and were not assigned human homologues. Similar GO analysis done using the available zebrafish gene names was less informative (data not shown).

A summary of the GO analysis at Biological Process level 4 of genes highly expressed in the pineal gland is shown in Figure 2. For this analysis, we selected at threefold enrichment, nonredundant probe sets for the three larval stages and for the two adult stages, both at day and night (Fig. 1), and converted these groups of genes to their human homologs. The most highly represented GO terms are related to photoreception (e.g., "visual perception" and "detection of light stimulus"), both in larval and adult stages. Terms for neuronal development and function ("neurotransmitter metabolism," "central nervous system development," and "transmission of nerve impulse"), "nitrogen compound biosynthesis," and "aromatic compound metabolism" were enriched at larval stages only, while GO terms related to programmed cell death and signal transduction ("intracellular signaling cascade" and "regulation of signal transduction") were found only at adult stages (Fig. 2). An additional GO analysis at Molecular Function level 4 showed that the terms "retinal," "retinol binding," and "G-protein coupled photoreceptor activity" were found prominently in all four samples (Supp. Fig. S2); again these terms suggest enrichment of genes related to visual perception.

Pathway analysis, as described in the Experimental Procedures section, identified "phototransduction pathway" as the most likely represented pathway in all larval and adult samples (Fig. 3). "Axonal guidance signaling" was also enriched in adult but less so in larval samples. Several signaling pathways were enriched only at adult stages, and among them "p53 signaling" was particularly interesting because of its involvement in programmed cell death which was found to be enriched in the GO analysis above (Fig. 2).

These analyses indicate that pineal gene expression is enriched in classes of genes among which those involved in phototransduction are most prominent, consistent with the function of the fish pineal gland as a photoreceptor organ. Furthermore, significant differences were found between gene sets enriched in larval and adult pineal glands.

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TABLE 2. Top 50 Pineal Gland Enriched Genes at Larval Stages[a]

	Probe set	Gene title	Gene symbol	P/B
1	Dr.352.1.S1	Floating head	flh	66.4
2	Dr.9835.1.S1	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 1	gngt1	57.5
3	AFFX-Dr-U43284-1_s	GFP	GFP	47.7
4	Dr.10292.1.S1	Retinol binding protein 4, like	rbp4l	37.4
5	Dr.9876.1.S1	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	gngt2	30
6	Dr.9829.1.S1	Phosphodiesterase 6G, cGMP-specific, rod, gamma	pde6g	26.5
7	Dr.9853.1.A1	Phosphodiesterase 6A, cGMP-specific, rod, alpha	pde6a	22.5
8	Dr.9908.1.A1	Similar to ENSANGP00000004777/LOC557454		21.7
9	Dr.12451.1.S1	Retinal pigment epithelium-specific protein a	rpepa	17.9
10	Dr.5738.1.S1	Similar to interphotoreceptor retinol-binding protein/LOC563355		17.7
11	Dr.12451.2.A1	Retinal pigment epithelium-specific protein a	rpepa	15.2
12	Dr.9899.1.S2	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	gnat1	12.7
13	Dr.8142.1.S1	Arylalkylamine N-acetyltransferase	aanat2	11.3
14	Dr.9871.1.A1	Recoverin	rcv1	9.5
15	Dr.8099.1.S1	Extra-ocular rhodopsin	exorh	9.4
16	Dr.12762.1.A1	Phosducin 2 / similar to Pdc2 protein/LOC100007784	pdc2	9
17	Dr.12469.1.S1	Arrestin 3, retinal (X-arrestin), like	arr3l	9
18	Dr.19931.1.S1	Tryptophan hydroxylase 1 (tryptophan 5-monooxygenase)	tph1	8
19	Dr.20586.1.A1	Similar to agouti related protein 2/LOC796595		7.6
20	Dr.15967.1.A1	Tryptophan hydroxylase 1 (tryptophan 5-monooxygenase)	tph1	7.4
21	Dr.9845.2.A1	ADP-ribosylation factor-like 3, like 2	arl3l2	7.1
22	Dr.9841.1.A1	Phosphodiesterase 6C, cGMP-specific, cone, alpha prime	pde6c	6.7
23	Dr.11240.1.A1	Similar to gefiltin/LOC555251		6.4
24	Dr.14052.1.A1	Tryptophan hydroxylase 2 (tryptophan 5-monooxygenase)	tph2	6.3
25	Dr.24898.1.S1	Chromosome 20 open reading frame 149 homolog (human)	c20orf149	6.3
26	Dr.11286.1.S1	Phospholipase A1 member A	pla1a	6.2
27	Dr.11085.1.A1	Retinaldehyde binding protein 1, like	rlbp1l	6
28	Dr.5167.1.A1	Similar to lambda-recombinase-like protein/LOC100004795		6
29	Dr.12902.1.A1	Cytochrome P450, family 11, subfamily B, polypeptide 2	cyp11b2	5.6
30	Dr.9899.1.S1	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	gnat1	5.6
31	Dr.10689.1.S1	zic family member 2 (odd-paired homolog, Drosophila) b	zic2b	5.5
32	Dr.6658.1.A1	Ca2+-dependent activator protein for secretion 2	cadps2	5.4
33	Dr.14325.1.S1	Cone-rod homeobox	crx	5.4
34	Dr.11305.1.A1	Guanylate kinase 1	guk1	5
35	Dr.12592.1.S1	Guanylate cyclase activator 1A	guca1a	4.9
36	Dr.17145.1.S1	Potassium channel tetramerization domain containing 12.1/similar to leftover/LOC796664	kctd12.1	4.9
37	Dr.16367.1.A1	Similar to centrin/LOC795513		4.6
38	Dr.9845.1.S1	ADP-ribosylation factor-like 3, like 2	arl3l2	4.5
39	Dr.16724.1.A1	Transcribed locus, strongly similar to NP_001076421.1 si:ch211-221n23.1		4.4
40	Dr.26347.1.A1	Pyrophosphatase (inorganic)	pp	4.3
41	Dr.26319.1.A1	Dopa decarboxylase	ddc	4.1
42	Dr.284.2.A1_a	Orthodenticle homolog 1	otx1	4.1
43	Dr.22887.1.A1	Similar to zinc finger protein Zic6/LOC796374		4
44	Dr.13970.1.S1	ADP-ribosylation factor-like 4a	arl4a	4
45	Dr.4807.1.S2	zic family member 2 (odd-paired homolog, *Drosophila*), a	zic2a	3.9
46	Dr.12624.1.S1_a	Iroquois homeobox protein 1, b	irx1b	3.9
47	Dr.10724.1.S1	Eomesodermin homolog a	eomesa	3.8
48	Dr.1730.1.A1	Similar to complement control protein factor I-B/LOC557557		3.6
49	Dr.9881.2.A1	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	gnat2	3.6
50	DrAffx.2.17.S1	Gamma-aminobutyric acid (GABA) B receptor, 1	gabbr1	3.5

[a]Values of P/B fold change for all six individual analyses (3d day, 5d day, 10d day, 3d night, 5d night, 10d night; see Supp. Table S1 for individual values) were averaged.

TABLE 3. Top 50 Pineal Gland Enriched Genes in Adult Stages[a]

	Probe set ID	Gene title	Gene symbol	P/B
1	Dr.10292.1.S1	Retinol binding protein 4, like	rbp4l	211.4
2	Dr.12592.1.S1	Guanylate cyclase activator 1A	guca1a	203.5
3	Dr.12469.1.S1	Arrestin 3, retinal (X-arrestin), like	arr3l	176.7
4	AFFX-Dr-U43284-1_s	GFP	GFP	169.7
5	Dr.12451.1.S1	Retinal pigment epithelium-specific protein a	rpepa	162.5
6	Dr.9908.1.A1	Similar to ENSANGP00000004777/LOC557454		154.6
7	Dr.9853.1.A1	Phosphodiesterase 6A, cGMP-specific, rod, alpha	pde6a	130.3
8	Dr.9841.1.A1	Phosphodiesterase 6C, cGMP-specific, cone, alpha prime	pde6c	128
9	Dr.9871.1.A1	Recoverin	rcv1	125.2
10	Dr.11305.1.A1	Guanylate kinase 1	guk1	119
11	Dr.8099.1.S1	Extra-ocular rhodopsin	exorh	110.8
12	Dr.9899.1.S1	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	gnat1	101.8
13	Dr.5738.1.S1	Similar to interphotoreceptor retinol-binding protein/LOC563355		94.7
14	Dr.9899.1.S2	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 1	gnat1	92.2
15	Dr.9876.1.S1	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 2	gngt2	90.9
16	Dr.9835.1.S1	Guanine nucleotide binding protein (G protein), gamma transducing activity polypeptide 1	gngt1	90.9
17	Dr.8071.1.S1	Opsin 1 (cone pigments), long-wave-sensitive, 1	opn1lw1	84.2
18	Dr.12762.1.A1	Phosducin 2/similar to Pdc2 protein/LOC100007784	pdc2	79.3
19	Dr.12451.2.A1	Retinal pigment epithelium-specific protein a	rpepa	78.1
20	Dr.352.1.S1	Floating head	flh	62.9
21	Dr.14052.1.A1	Tryptophan hydroxylase 2 (tryptophan 5-monooxygenase)	tph2	58.5
22	Dr.8142.1.S1	Arylalkylamine N-acetyltransferase	aanat2	55.5
23	Dr.24898.1.S1	Chromosome 20 open reading frame 149 homolog (human)	c20orf149	49.5
24	Dr.15426.1.S1	Orthodenticle homolog 5	otx5	47.8
25	Dr.19931.1.S1	Tryptophan hydroxylase 1 (tryptophan 5-monooxygenase)	tph1	43.7
26	Dr.11085.1.A1	Retinaldehyde binding protein 1, like	rlbp1l	41.8
27	Dr.1730.1.A1	Similar to complement control protein factor I-B/LOC557557		40.1
28	Dr.20586.1.A1	Similar to agouti related protein 2/LOC796595		38.4
29	Dr.25442.1.A1	Elongation of very long chain fatty acids (FEN1/Elo2, SUR4/Elo3, yeast)-like 4	elovl4	35.5
30	Dr.15967.1.A1	Tryptophan hydroxylase 1 (tryptophan 5-monooxygenase)	tph1	33.4
31	Dr.9845.2.A1	ADP-ribosylation factor-like 3, like 2	arl3l2	33.3
32	Dr.2377.1.A1	Keratin, type 1, gene 19d	krt1-19d	31.9
33	Dr.14325.1.S1	Cone-rod homeobox	crx	31.9
34	Dr.26319.1.A1	Dopa decarboxylase	ddc	29
35	Dr.9881.1.S1	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	gnat2	25.5
36	Dr.20115.1.S1	Cofilin 1 (non-muscle)	cfl1	25.4
37	Dr.2377.2.S1	Keratin, type 1, gene 19d	krt1-19d	24.4
38	Dr.26347.1.A1	Pyrophosphatase (inorganic)	pp	23.8
39	Dr.9881.2.A1	Guanine nucleotide binding protein (G protein), alpha transducing activity polypeptide 2	gnat2	22.8
40	Dr.4833.1.S1	Annexin A1c/zgc:86853	anxa1c	21.7
41	Dr.19801.1.A1	Similar to fatty acid binding protein 1b/LOC795525		21.4
42	Dr.994.1.S1	Claudin b	cldnb	21.3
43	Dr.24943.1.S1	Similar to Desmoglein 2/LOC560026		21.3
44	Dr.11283.1.A1	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3, like	slc25a3l	21.1
45	Dr.25140.7.A1_a	Tumor-associated calcium signal transducer/zgc:110304/ protein LOC791868/similar to pan-epithelial glycoprotein/LOC100000093	tacstd	20.9
46	Dr.6924.1.S1	S-adenosylhomocysteine hydrolase	ahcy	20.4
47	Dr.3499.3.A1	Coronin, actin binding protein, 1A	coro1a	19.1
48	Dr.1434.1.S1	Keratin 5	krt5	18.5
49	Dr.25556.1.S1	Keratin 15	krt15	18
50	Dr.9829.1.S1	Phosphodiesterase 6G, cGMP-specific, rod, gamma	pde6g	18

[a]Adult stages (see Supp. Table S2 for individual values) were analyzed as described in Table 2.

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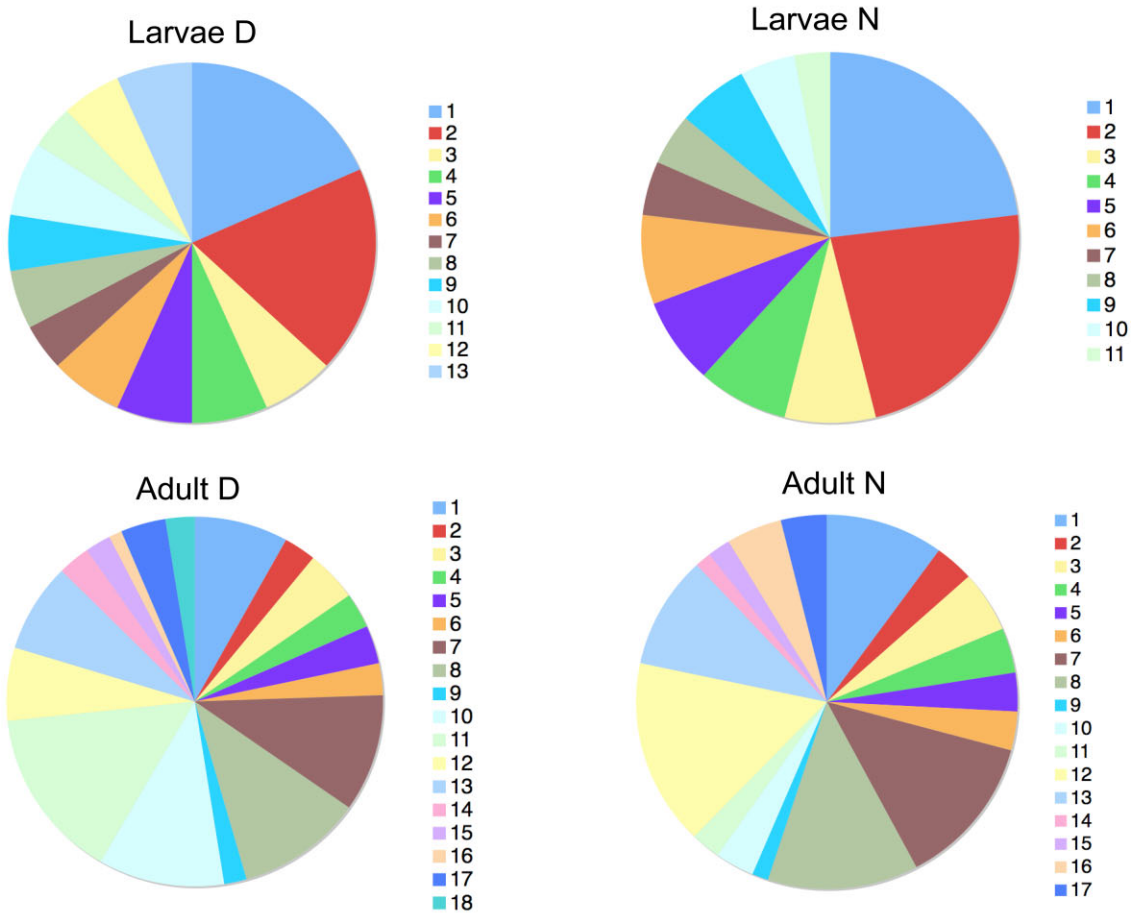


GO term	Larvae D	Larvae N	Adult D	Adult N
visual perception	4.13E-15 (1)	6.01E-16 (1)	2.96E-09 (1)	3.95E-10 (1)
detection of light stimulus	7.02E-07 (3)	1.10E-06 (3)	4.98E-06 (2)	4.78E-06 (2)
epidermis development		0.0347 (8)	7.38 E-06(3)	6.92E-06 (3)
response to light stimulus	5.65E-06 (6)	8.83E-06 (6)	9.64E-06 (4)	1.01E-04 (6)
detection of external stimulus	5.65E-06 (5)	8.83E-06 (5)	9.64E-06 (5)	9.20E-06 (4)
detection of abiotic stimulus	3.59E-06 (4)	5.60E-06 (4)	5.50E-05 (6)	5.29E-05 (5)
programmed cell death			2.27E-04 (7)	0.0205 (13)
negative regulation of cellular physiological process			7.52E-04 (8)	6.75E-04 (7)
calcium-independent cell-cell adhesion			9.08E-04 (9)	
sensory perception	1.77E-07 (2)	9.85E-08 (2)	0.00334 (10)	0.00303 (8)
intracellular signaling cascade			0.00468 (11)	0.0201 (12)
regulation of programmed cell death			0.0068 (12)	
organic acid metabolism	0.0716 (13)		0.00715 (13)	
sensory perception of sound			0.0154 (14)	0.01489 (10)
vitamin metabolism			0.0196 (15)	0.0192 (11)
myoblast differentiation			0.0318 (16)	
regulation of signal transduction			0.0422 (17)	0.0406 (16)
positive regulation of signal transduction			0.0491 (18)	
nitrogen compound biosynthesis	0.0218 (11)	0.0268 (7)		
amino acid and derivative metabolism	0.0680 (12)			
aromatic compound metabolism	0.00578 (8)	0.0628 (10)		
epithelial cell differentiation				0.00574 (9)
cell fate determination				0.0357 (14)
negative regulation of transferase activity				0.0379 (15)
regulation of cell size				0.0408 (17)
neurotransmitter metabolism	0.00193 (7)	0.0708 (11)		
central nervous system development	0.00634 (9)			
transmission of nerve impulse	0.00765 (10)	0.0570 (9)		

Fig. 2. GO term analysis of pineal-enriched genes at biological processes (BP) level 4. GO terms with *P* values < 0.1 (larvae) and < 0.05 (adult) were selected. Nonoverlapping genes were considered (Fig. 1). D, day, and N, night. The width of each slice indicates the number of genes in a given GO term. The numbers next to the colored squares indicate the GO terms, arranged by *P* values (small to large), corresponding to the numbers in parentheses in the table below. These *P* values indicate the probability of finding this GO term occupancy by chance, with a blank space indicating that the GO term was not enriched in the pineal gland. The color scheme starts at 12 o'clock and proceeds clockwise.

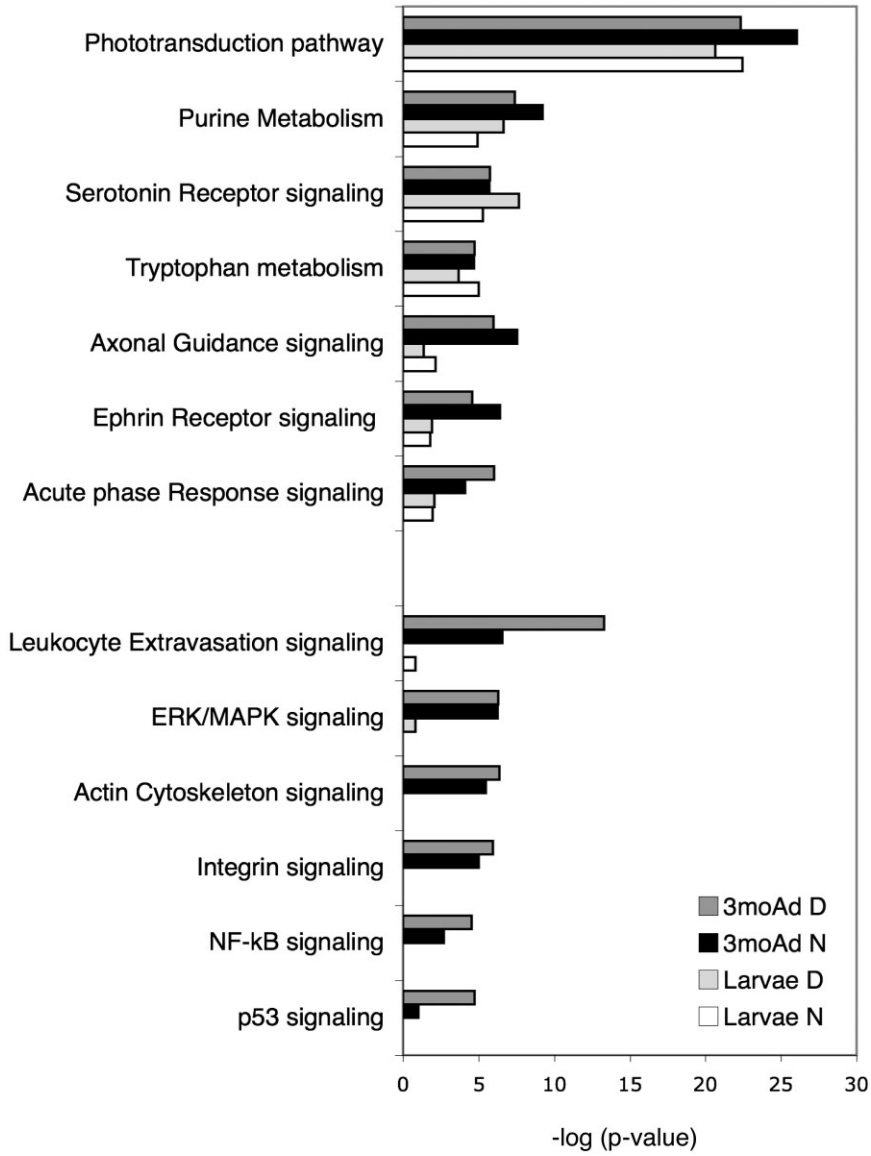


Fig. 3. Canonical pathways enriched in the pineal gland. Pathways enriched in all four samples (larvae day [D] and night [N], adult day [D] and night [N]) are shown at the top. The pathways shown at the bottom are enriched mainly in the adult pineal gland.

Transcriptome Analysis of Developmentally Regulated Genes

Developmental changes in pineal gene expression were identified by searching for genes that exhibited a ≥ threefold difference between the highest and lowest signal value among the five stages examined (3 days, 5 days, 10 days, 3 months, and 1–2 years), having a signal ≥ 200 in at least one stage, and P value ≤ 0.05. This analysis does not consider enrichment in the pineal relative to the brain. Using these criteria, 2,370 probe sets were selected from daytime pineal gland data as changing during development. Cluster analysis was performed with these probes, and the set was divided into four subclusters (Fig. 4). In broad terms, subsets A and B contain genes whose expression levels are low during larval stages but increased at adult stages, while subsets C and D show the opposite profile. GO analysis of clusters A and C was done, while subsets B and D contain too few genes for this analysis. Subsets A and C, which contained 1,191 and 733 zebrafish probe sets, respectively, were converted to 552 and 299 human homologs as indicated above. The results of GO analysis at Biological Process level 5 and Molecular Function level 3 are shown in Figure 5 and Supplementary Figure S3, respectively.

Similar analysis with the pineal gland "night" samples produced essentially similar results (Supp. Figs. S4, S5, S6).

In subset A, GO terms related to phototransduction were considerably enriched (Fig. 5; Supp. Figs. S3, S5, S6). Furthermore, terms related to cell death are also represented (Fig. 5; Supp. Fig. S5). These GO terms were found in genes enriched in the adult pineal gland, reflecting physiological function rather than development of the pineal gland. In contrast, terms related to transcription were highly enriched in subset C (Fig. 5; Supp. Figs. S3, S5, S6). Likewise, GO terms such as "brain development" and "neuron differentiation" were found in genes enriched at larval stages (Fig. 5; Supp. Fig. S5). These terms clearly reflect developmental changes that occur during these stages.

To illustrate the classes of genes that represent the different categories we chose GO terms that were most significantly represented in each subset. For each of these terms, we show the genes included as hierarchical clusters in Figures 6 (subset A) and 7 (subset C). It is apparent that groups of genes included in certain GO terms are co-regulated at different stages in the pineal gland.

Analysis of Genes That Exhibit Night/Day Expression Differences in the Pineal Gland

The mid-day and mid-night gene expression profiles of the pineal gland were compared at different developmental stages. Although this approach does not necessarily reflect circadian clock control of gene expression and is likely to miss changes that do not peak at midday or midnight, it provides a useful approach to characterizing daily changes in gene expression.

Probe sets were selected using the following criteria: P value ≤0.05 and night and day signal ratio ≥1.5 or 2 (Fig. 8). Approximately 250 genes were identified that showed at least

Developmental Dynamics

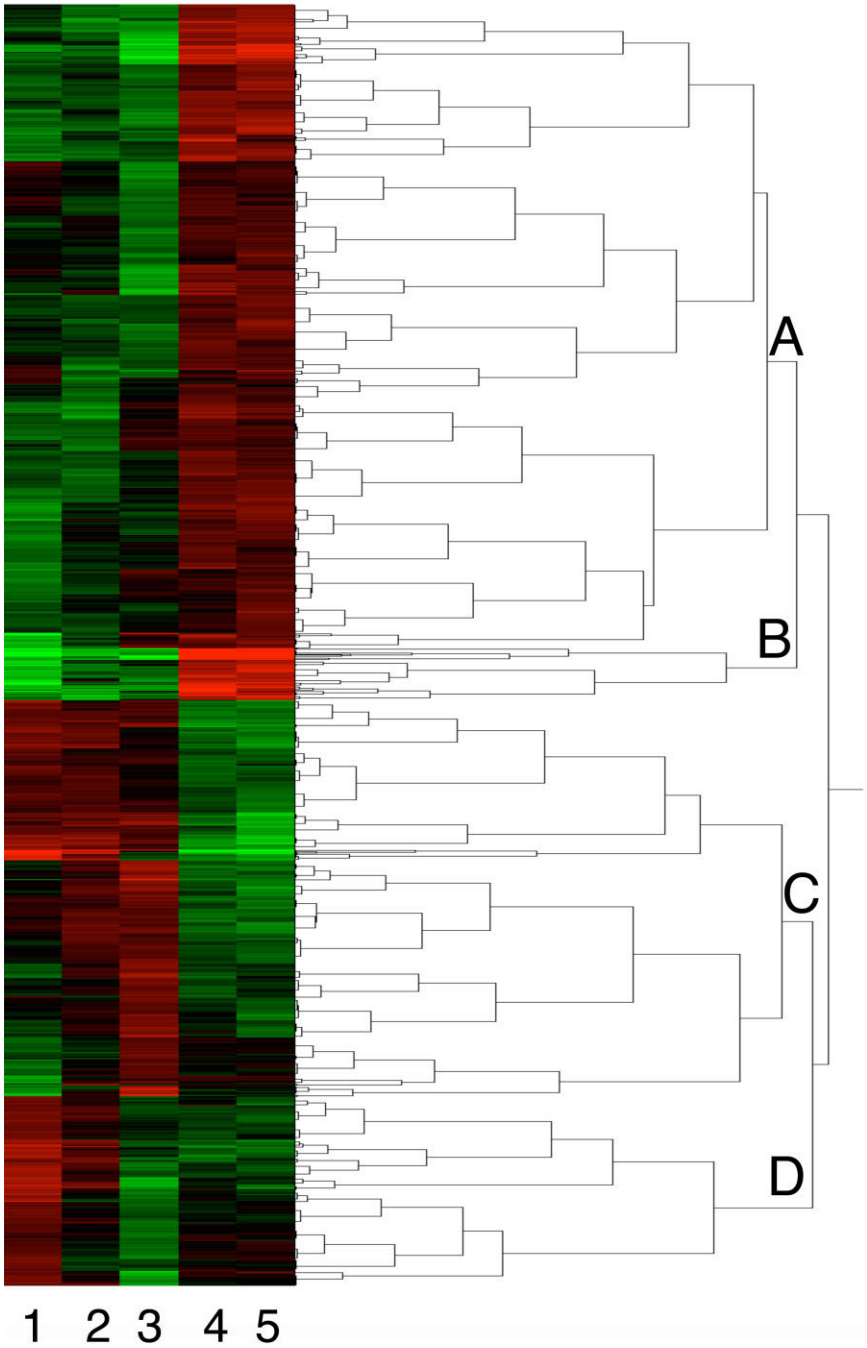


Fig. 4. Hierarchical clustering of genes whose expression levels change during the day in the pineal gland during development. Genes were selected using the following criteria: average maximum signal ≥200; lowest-to-highest signal ratio ≥ 3; *P* value ≤0.05. Low expression: green; high expression: red. Samples were grouped into four subsets, A–D. 1, 3 days; 2, 5 days; 3, 10 days; 4, 3 months; 5, 1–2 years.

≥1.5-fold differences in expression between day and night in four of the five stages tested. There was no significant difference in the number of genes selected between larvae at d5 and d10 and adults at 3 months and 1–2 years, however, a higher number of genes exhibited day/night differences at larval stage d3. The biological meaning of this result remains to be elucidated.

The probe sets showing day/night differences in at least four of the five developmental stages are listed in the Supplementary Table S3. *aanat2* was selected as a gene highly expressed at night, as expected. Another gene highly expressed at night is bHLH domain containing class B, an inhibitor of the BMAL:CLOCK circadian transcription activator. The circadian clock gene *per2* and *rbp4* (retinal binding protein 4), which was also identified as highly expressed in the pineal gland compared with the brain, were selected as being highly expressed during the day. These results further strengthen the validity of the microarray analysis and the genes identified here.

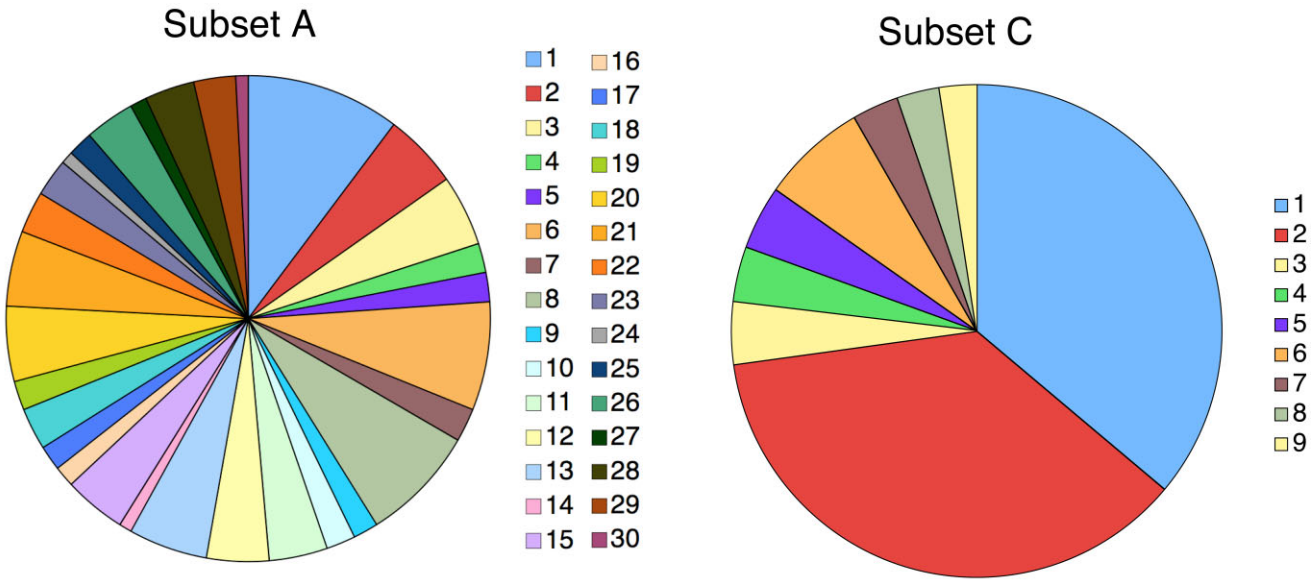
unc119 Homolog Is Highly Enriched in the Pineal Gland

Unc119 homolog (Dr. 9908, Supp. Table S1) was identified as a gene of special interest because it is highly expressed in the pineal gland at both day and night compared with the brain at all stages we examined, and might play a role in pineal gland development and/or function. Because this is the third family member of *unc119* found in the zebrafish, we refer to this gene as *unc119c*.

Unc119c shares 45.7% amino acid identity with the previously published zebrafish Unc119 (Manning, 2004) and is clearly most diverged from Unc119 homologues in other species (data not shown). In situ hybridization experiments revealed that *unc119c* is expressed in the pineal gland at 72 hpf (Fig. 9A). The transcript is not found at the 20 somite stage or earlier (data not shown).

Recently, ADP-ribosylation factor-like protein 2 (ARL2) has been identified as an interacting protein of a human homologue of UNC119, HRG4 (Kobayashi et al., 2003). Of interest, members of the *arl* gene family, *arl3l1* (ADP-ribosylation factor-like protein 3 like 1) and *arl3l2*, were highly enriched in the pineal gland relative to brain in our microarray data. *arl3l2* is expressed in the zebrafish pineal gland, as visualized by in situ hybridization (Fig. 9B), suggesting a possible interaction between Unc119c and Arl3l2.

To test the possibility that Unc119c and Arl312 interact, we performed co-immunoprecipitation studies with FLAG-tagged Unc119c and myc-tagged zebrafish Arl3l2 in cultured cells. Arl3l2 specifically interacted



sub A	GO term	P-Value
1	carboxylic acid metabolism	2.22E-08
2	fatty acid metabolism	1.13E-06
3	sensory perception of light stimulus	1.18E-05
4	phototransduction	1.43E-05
5	detection of light stimulus	1.89E-05
6	cellular lipid metabolism	0.00103402
7	organic acid biosynthesis	0.00110235
8	apoptosis	0.00139626
9	regulation of actin polymerization and/or depolymerization	0.00238733
10	fatty acid biosynthesis	0.00248673
11	regulation of protein metabolism	0.00394048
12	coenzyme metabolism	0.00428665
13	cellular macromolecule catabolism	0.0048168
14	detection of visible light	0.00622689
15	amino acid metabolism	0.0072356
16	negative regulation of cell organization and biogenesis	0.00773029
17	actin polymerization and/or depolymerization	0.00813837
18	regulation of protein kinase activity	0.00833844
19	nitrogen compound catabolism	0.00838947
20	regulation of apoptosis	0.01096621
21	regulation of programmed cell death	0.01152681
22	negative regulation of programmed cell death	0.01238588
23	cation homeostasis	0.01511967
24	antigen receptor-mediated signaling pathway	0.02094551
25	amine catabolism	0.02846669
26	lipid biosynthesis	0.03058862
27	hormone biosynthesis	0.03408209
28	nucleotide metabolism	0.03508386
29	cofactor biosynthesis	0.03649043
30	fat-soluble vitamin metabolism	0.03650811

sub C	GO term	P-Value
1	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metaboli	2.55E-07
2	transcription	3.43E-07
3	brain development	1.45E-05
4	neuron differentiation	0.01136353
5	negative regulation of cell proliferation	0.01852005
6	cytoskeleton organization and biogenesis	0.02149553
7	proton transport	0.03706173
8	hydrogen transport	0.0383163
9	negative regulation of protein metabolism	0.04726615

Fig. 5. GO term analysis of subsets A and C of Figure 4 at biological processes (BP) level 5. GO terms which *P* values ≤0.05 were selected. The numbers next to the colored squares correspond to the numbers in the table below; see also legend to Figure 2.

Developmental Dynamics

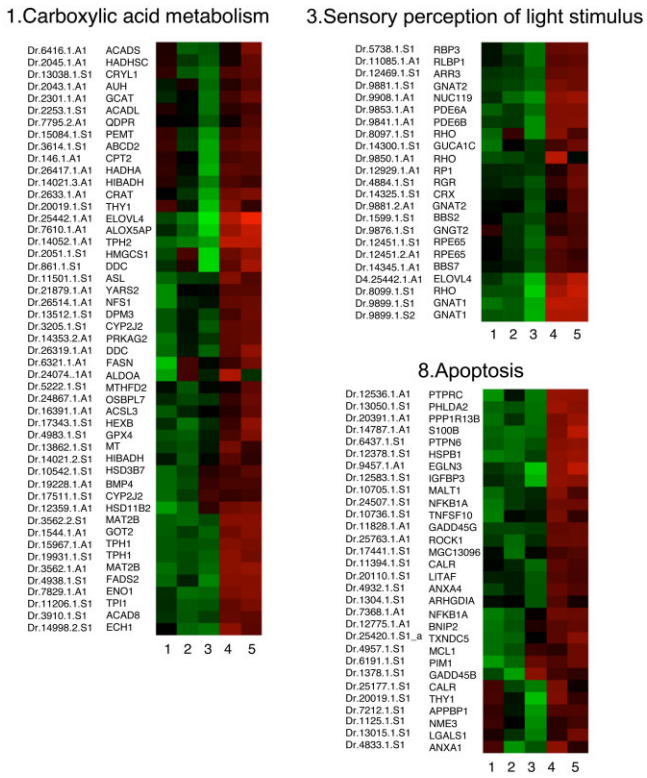


Fig. 6. Clustering of genes in the selected GO terms shown in Figure 5, subset A. Each row of the heat map (see Fig. 4 for explanations) corresponds to one probe set. Affymetrix probe set ID numbers and gene symbols are shown on the left. 1, 3 days; 2, 5 days; 3, 10 days; 4, 3 months; 5, 1–2 years.

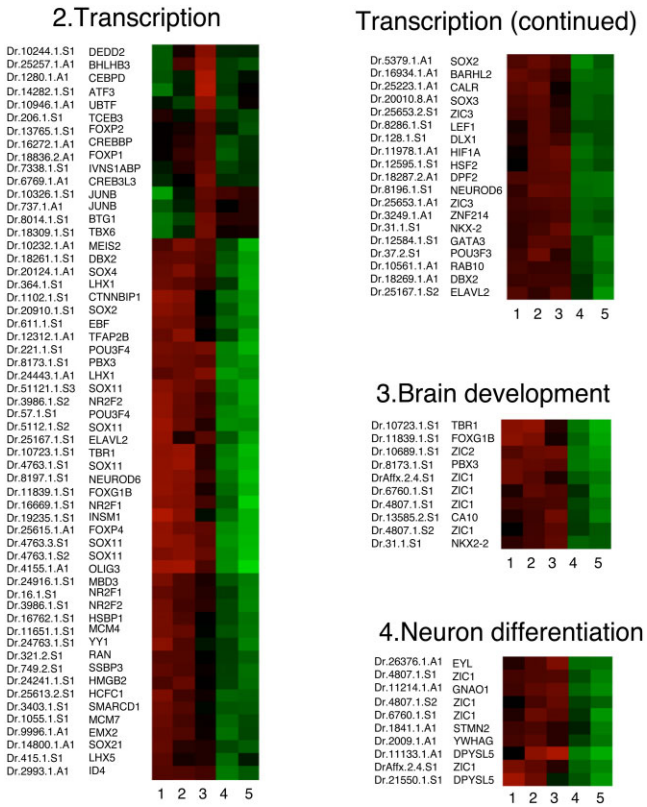


Fig. 7. Clustering of genes in the selected GO terms shown in Figure 5, subset C. Each row of the heat map corresponds to one probe set. Affymetrix probe set ID numbers and gene symbols are shown on the left. 1, 3 days; 2, 5 days; 3, 10 days; 4, 3 months; 5, 1–2 years.

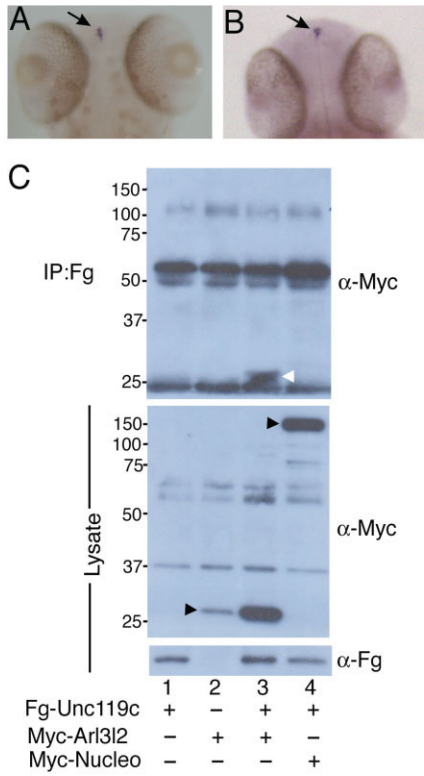


Fig. 9. *unc119c* and *arl3l2* are co-expressed in the pineal gland and interact with each other. **A,B:** Spatial expression of zebrafish *unc119c* (A) and *arl3l2* (B). Both panels are shown as dorsal views of 3 dpf larvae. In situ hybridization was carried out as described (Toyama and Dawid, 1997). **C:** Co-immunoprecipitation of zebrafish Unc119c and Arl3l2 transfected into HEK293 cells. The antibody used for precipitation is indicated by IP, and the antibodies used for blotting are shown on the right side of the panels. Myc-tagged Arl3l2 co-precipitated with Flag-Unc119c (white arrowhead in the top panel, shown above immunoglobulin light chain.), but Myc-tagged nucleolin (Myc-Nucleo) did not. Black arrowheads indicate Myc-tagged Arl3l2 and Nucleolin (used as negative control) in the middle panel.

with Unc119c (Fig. 9C). This suggests that these two proteins may be part of a common functional pathway in the zebrafish pineal gland.

DISCUSSION

Genes Highly Expressed in the Pineal Gland

In this study, we have systematically examined the gene expression profiles in the larval and adult zebrafish pineal gland. To identify genes whose expression is enriched in the pineal gland, we used brain tissue without pineal gland and without eyes as reference. Eyes were removed from con-

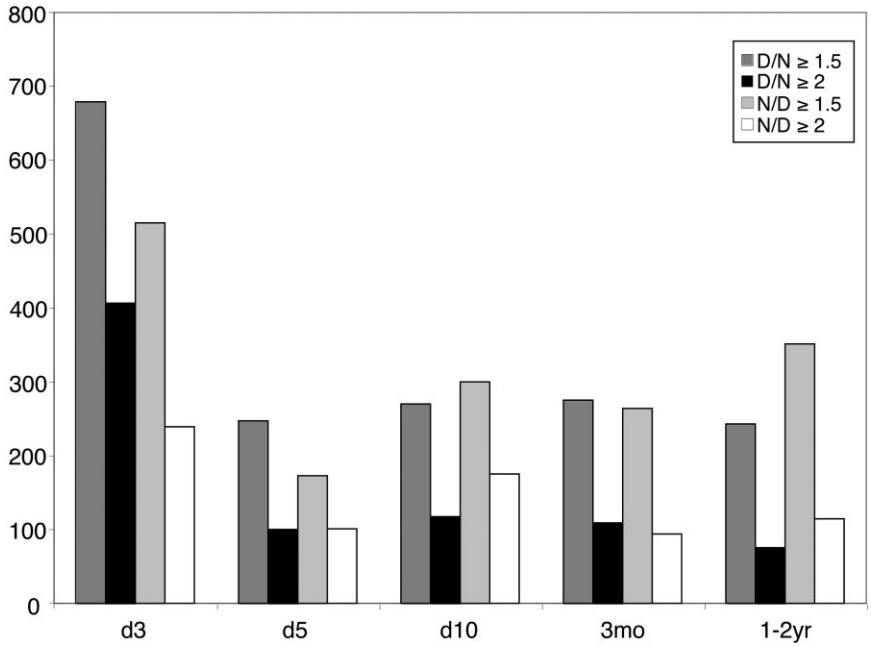


Fig. 8. Number of genes highly expressed at day or night in the pineal gland. Samples were analyzed at each developmental stage separately. The following criteria were used for the selection of probe sets: Average minimum signal ≥100; *P* value ≤0.05. Dark gray: D/N≥1.5; black: D/N≥2; light gray: N/D≥1.5, white: N/D≥2.

trol samples because the zebrafish pineal gland is a photoreceptive organ and, therefore, similar molecular pathways may be active in eyes and pineal gland.

While there is significant overlap between genes highly expressed in the pineal gland at larval and adult stages, we considered the two groups of data separately for GO analysis. This approach revealed the enrichment of developmentally specific GO terms which would be obscured by combining all the data. As result, we found that GO terms for neuronal development and function were specifically enriched at larval stages.

Throughout this microarray analysis, we obtained evidence for many genes that are highly expressed in the pineal gland relative to brain. At present, nearly half of the probe sets are not well annotated, being referred to as "transcribed locus" or "hypothetical protein." Future expansion of the EST database and completion of the zebrafish genome will promote the analysis of these genes. Nevertheless, we generated a list of many annotated genes which have not been reported previously as expressed in the pineal gland. Individual analysis of some of these genes, and of genes that may be annotated in the future, offer the opportunity to identify new players in pineal development and function. Some of these newly identified pineal genes may relate to the establishment of asymmetry in the pineal and surrounding epithalamic region (Harris et al., 1996; Concha and Wilson, 2001; Gamse et al., 2003; Snelson et al., 2008). This proposal is supported by the finding that *otx5*, which is enriched in the pineal gland, also exhibits asymmetric expression in the zebrafish epithalamus.

The finding of a highly expressed transcript in the pineal gland provides reason to characterize unannotated genes because such highly expressed genes are likely to be functionally important. In addition to the *unc119* homologue (see below), another interesting novel gene is the zebrafish homolog of *agrp* which corresponds to probe set Dr. 20586.1.A1 (Supp. Table S1). AgRP is a key hypothalamic regulator of ingestive behavior in mammals and zebrafish (Song et al., 2003). An immunocytochemical study in zebrafish using an antibody against human AgRP revealed unexpected AgRP immunoreactivity in the pineal gland, leading to the suggestion that this was due to the expression of the gene in the pineal gland termed AgRP2 (Forlano and Cone, 2007). Here, we independently reached the same conclusion based on our microarray analysis. The pineal-enhanced expression of the AgRP2 homolog suggests a possible connection between the circadian oscillator in the zebrafish pineal to ingestive behavior, an attractive direction for further research.

Previous Microarray Studies on Gene Expression in the Vertebrate Pineal Gland

Three groups have previously published results of microarray analysis of the rat pineal gland (Humphries et al., 2002; Bailey et al., 2008; Fukuhara and Tosini, 2008). Fukuhara et al. found that approximately 2% of a total of 8,000 genes on the microarray showed rhythmic expression in the pineal gland, consistent with the findings by Humphries et al. (approximately 3% out of 1,176 genes). Bailey et al. found that approximately 4% of the genes (~600 of ~13, 000) exhibit greater than twofold change on a night/day basis. Our data identified approximately 500 genes which are up-regulated at day or night in the adult zebrafish pineal gland (approximately 3% of all genes on the microarray). Our finding agrees with previous observations (Humphries et al., 2002; Bailey et al., 2008; Fukuhara and Tosini, 2008) that only a limited number of genes exhibited day/night differences in their expression level.

All previous analyses were done with adult rat pineal gland and found that more genes were up-regulated at night as compared to those up-regulated during the day (47 vs. 13; Fukuhara et al., 2008). Likewise, Bailey et al. found approximately twofold more genes that were elevated at night (Bailey et al., 2008). In contrast, we found similar numbers of genes up-regulated at day and at night. Furthermore, we identified many more genes showing day/night differences in expression, which had not been reported previously. Only few genes previously reported to display a diurnal rhythm of expression were identified in our analysis. One of the genes characterized by Humphries et al. (2002) as a nocturnal up-regulated gene, *Id-1* (inhibitor of DNA binding and differ-

entiation), did not show significant day/night changes in its expression in the zebrafish pineal gland. These differences may be caused by species differences or by incomplete annotation of the zebrafish microarray.

Possible Functional Interaction Between Unc119c and Arl3l2

The *unc119* gene is a new class of neural gene that shares conserved sequences in all metazoans examined. Although this gene is highly conserved from worms to human, its expression pattern shows two extremes. In invertebrates (*C. elegans* and *Drosophila*), this gene is expressed throughout the nervous system, and *unc119* knockout causes damage to neurons widely distributed in the nervous system (Maduro et al., 2000; Knobel et al., 2001). In mammals (human, mouse, rat), this gene was originally identified as *HRG4* (human retinal gene 4) (Higashide et al., 1996), a gene that is expressed specifically in the photoreceptor synapse. A truncation mutation of HRG4 is associated with late-onset cone-rod dystrophy in humans, and transgenic mice containing the same mutation, develop late-onset retinal degeneration (Kobayashi, 2000). *HRG4* knockout in the mouse causes severe damage to the retina (Ishiba et al., 2007). In zebrafish, one *unc119* homolog has been identified (*unc119*) with an expression pattern similar to that seen in invertebrates, i.e., expression throughout the central nervous system; its knock down results in disorganized neural architecture (Manning et al., 2004). A second *unc119* homolog (*unc119b*) was found in the zebrafish genome but its expression pattern was not characterized (Manning et al., 2004). Here, we show the existence of a third *unc119* homolog (*unc119c*) that exhibits enhanced expression in the pineal gland.

Although the function of UNC119 is not clearly understood, the recent finding that ARL2 (ADP-ribosylation factor-like protein 2) interacts with HRG4 suggests a possible function for UNC119 in the retina. ARL2 is a guanine nucleotide binding protein and may play a role in microtubule assembly (Radcliffe et al., 2000). ARL2 interacts with BART (binder-of-ARL2), enabling it to enter mitochondria and bind ANT-1 (adenine nucleotide transporter), which is thought to be involved in apoptosis (Mori et al., 2006). Therefore, a truncation mutation of HRG4 may lead to mitochondrial ANT-1-mediated retinal degeneration by apoptosis through ARL2.

Here, we demonstrate the coexpression of *unc119c* and *arl3l2*, one of the closest homologs of *arl2*, as well as the physical interaction between Unc119c and Arl3l2. To our knowledge, this is the first evidence suggesting an Unc119-Arl interaction in the pineal gland. Recently, Veltel et al. reported the formation of a ternary complex between Arl3, its GAP RP2 (retinitis pigmentosa 2), and HRG4 (Veltel et al., 2008). Also, mouse Unc119 interacts with the synaptic ribbon specific protein RIBEYE at photoreceptor ribbon synapses (Alpadi et al., 2008). The previously reported functions for UNC119 are all related to its expression in the retina. However, Bailey et al. also identified Unc119 as a highly expressed gene in the pineal gland and the retina relative to other tissues (Bailey et al., 2008), and our findings indicate that a distinct tissue-specific Unc119 homolog may play a role in photoreceptor cell function in the pineal gland. The high expression of Unc119 in photoreceptors might be a conserved feature in vertebrates, possibly based on similar functions. Whether the existence of an additional *unc119* homolog is a unique feature of the zebrafish is not known, but it should be remembered that the zebrafish pineal gland is located superficially and contains photoreceptor cells, in contrast to the situation in higher vertebrates. Zebrafish may, therefore, have gained an additional *unc119* gene that is functional outside of the retina.

EXPERIMENTAL PROCEDURES

Collection of Larvae and Adult Pineal Glands and RNA Preparation

Adults and larvae were kept under a 14-hr-light/10-hr-dark cycle. Pineal glands were isolated manually using a fluorescence dissection microscope, guided by green fluorescent protein (GFP) fluorescence, from larval (3 days, 5 days, and 10 days) and adult (3 month and 1–2 years) transgenic zebrafish in which expression of the GFP gene is driven by the *aanat2* promoter (Gothilf et al., 2002). For comparison, brain tissue from which the pineal gland and eyes had been removed was also collected (referred to as "brain" from here onward). The tissues were collected directly in QIAzol (Qiagen). Five to nine adult or 10 to 38 larval pineal glands, and 2 to 3 adult or 2 to 5 larval brains were pooled to yield one sample. Three to five samples were collected at midday and midnight at each developmental stage.

Total RNA was prepared using the RNeasy Lipid Tissue Mini Kit (Qiagen) and biotin-labeled cDNA was generated using the Ovation Biotin system kit (NeuGen)

Microarray Analysis and Data Processing

The Affymetrix GeneChip Zebrafish Genome Array was hybridized and processed using the standard Affymetrix protocol. Altogether, we collected 20 types of samples: five time points (3 days, 5 days, 10 days, 3 months, and 1–2 years), two organs (pineal gland and brain), and two sampling times (day and night). For each type of sample, tissue was obtained and processed three to five times. After hybridization, microarray chips were scanned, quantitated, and normalized by GCOS (Affymetrix). All data were submitted to the NCBI GEO database as series GSE13371. There are 15,617 probe sets on a zebrafish Affymetrix microarray chip, including 114 hybridization controls; data for the remaining 15,503 probe sets were subjected to further statistical using JMP, the statistical Discovery Software (http://www.jmp.com/). Unless otherwise indicated, three to five replicates for each sample type were averaged, and probe sets showing differences with a P value smaller than 0.05 were considered further.

Gene Ontology (GO) Analysis

To take advantage of the more complete annotation of the human genome, we converted lists of selected zebrafish genes to those of their hu-

man homologs, using the WEB site of the Zebrafish Gene and Microarray Annotation Project (ZGMAP) (Children's Hospital Boston, http://134.174.23.160/zfACA/hash/cumulative_expanded.aspx). GO term analysis was performed using the DAVID Bioinformatics Resources, NIAID/NIH, with the default level of $P < 0.05$ to select genes to be included, unless noted otherwise.

Pathway Analysis

Ingenuity Pathways Analysis software (http://www.ingenuity.com/products/pathways_analysis.html) (ver.5) was used to identify canonical pathways most likely to be active in the pineal gland. Selected zebrafish genes were converted to a list of human genes as indicated above before this analysis.

Immunoprecipitation

HEK-293 cells were transfected with pcDNA3Unc119c-Flag, pcDNA3Arl3l2-Myc, and pcDNA3Nucleolin-Myc. Proteins were extracted and precipitated with anti-Flag antibodies (Sigma, F3165) coupled to anti-mouse IgG agarose beads (Sigma, A6531).

Proteins were detected with anti-Myc (Sigma, C6594) or anti-Flag (Sigma, F3165) antibodies.

ACKNOWLEDGMENTS

We thank Mark Rath for excellent technical assistance. This work was partially supported by a grant from the United States-Israel Binational Science Foundation (BSF), Jerusalem, Israel, and by the Intramural Research Program of the NICHD, NIH.

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